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PRINCIPAL INVESTIGATOR: Shafiq Khan, Ph.D.

CONTRACTING ORGANIZATION: Clark Atlanta University
Atlanta, Georgia 30314

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Shafiq Khan, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Clark Atlanta University
Atlanta, Georgia 30314

E-Mail: shafiq.khan@ttmc.ttuhsc.edu

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13. ABSTRACT (Maximum 200 Words)

In order to identify surface antigens that are differentially expressed in prostate cells during progression from normal to the cancerous and metastatic states, we have sought to use antibody phage display and paired cell lines that differ in tumorigenic and metastatic potential. In this method affinity subtraction and purification selects recombinant monoclonal antibodies binding to antigens that differ between closely related cell types, even without prior knowledge of the nature of the antigens involved. Collaborator Dr. Leland Chung at the Winship Cancer Institute developed lineage-related human prostate cancer cell lines, LNCaP and C4-2, that differ in metastatic potential. Research associates are being trained in a variety of techniques towards this goal. New collaborators Dr. Shafiq Khan and Dr. Sean Kimbro have been involved, and the PI is seeking training in improved methods for isolation of protein affinity reagents. Alternative strategies for identifying metastasis-associated biomarkers have been undertaken, including immunochemical screening for know growth factor receptors and responses , as well as differential transcription by microarray-based gene discovery techniques.

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Biomarkers for Metastatic Prostate Cancer

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INTRODUCTION:

The project involves the isolation of antibodies that identify molecules associated with the transition of human prostatic epithelium to metastatic adenocarcinoma. An immunochemical test for prostate specific antigen (PSA) has been valuable for early diagnosis of prostatic abnormalities including prostate adenocarcinoma (PCa). However, the PSA test does not distinguish benign from life-threatening disease, and no corresponding test for progression of the disease exists. In order to **identify surface antigens** that are differentially expressed in prostate cells during cancer progression we will use **antibody phage display** and paired cell lines that differ in **metastatic potential**. In this method, combining affinity subtraction with affinity purification selects MAbs that bind to antigens that differ between closely related cell types, even without prior knowledge of the nature of the antigens involved. Collaborator Dr. Leland Chung at the Winship Cancer Institute developed lineage-related human prostate cancer cell lines, LNCaP and C4-2B, that differ in metastatic potential [1] and by inference in the presence of metastasis-associated antigens. The specific aims of the research are to *1)* Isolate novel MAbs that bind to metastatic C4-2B cells more efficiently than to non-metastatic LNCaP cells; *2)* Evaluate the efficacy of the MAbs in inhibiting growth, migration, attachment and invasion by human prostate cancer cell lines; *3)* Correlate the presence and distribution of the antigens identified by the MAbs with patient specimens of known prognosis.

BODY:

Personnel

Several personnel transitions have taken place during the year. Technicians Bernadette Jean-Joseph and Tony Griffin moved on the higher paying positions at Emory University and CAU respectively. Technician Soma Sannigrahi was brought on half time in December and will be converted to full time beginning July. Graduate student Sabrenia Parker worked with us over summer 2003 and student Aron Tesfamichael left the group. The bulk of the work described was conducted by Drs. Jamil Haider and Tea Okou. Eminent scholar Dr. Shafiq Khan was recruited to CAU in January, and with two experienced technicians, has joined our weekly Thursday morning group meetings. Dr. Sean Kimbro and technical associate Ms. Jody Moore are leading the microarray-based biomarker discovery project. Towards selection of recombinant proteins that bind specifically to prostate cancer cells, P.I. Williams has accepted a mid-career fellowship at the National Human Genome Research Institute-sponsored Molecular Science Institute (MSI) in Berkeley, CA. This training fellowship should dramatically enhance our capability to achieve the goals of the USAMRMC project upon his return. MSI president Dr. Roger Brent was one of the developers of the two-hybrid method for discovery of protein-protein interactions [2]. Using a variety of selection platforms (M13 gene 3, T7 gene 10, flagellar, yeast surface, etc), affinity protein scaffolds (scFv, TrxA, lectin, forkhead domain) and selection protocols, we will develop methods that permit rapid, at-will selection of protein affinity reagents against targets which may be of the varied chemical natures known to be relevant in cancer associated antigens (e.g. carbohydrate, lipid and phosphate as well as protein).

Phage Display Subtraction And Selection On Cultured Cells

We have performed subtraction and selection of the Tomlinson antibody scFv libraries [3] on LNCaP and C4-2 cells. Two different selection protocols, each comprising 3 selection cycles, were performed. Each protocol used ~ 10^7 LNCaP and C4-2 cells that had been detached from the plastic substratum using EDTA, and washes we conducted by low speed centrifugation. The first protocol used LNCaP subtraction followed by C4-2 selection, while the 2nd protocol used a three step C4-2 selection/LNCaP subtraction/C4-2 selection. Because of difficulties encountered in prior screens using immobilized whole prostate cells, we have instead used cell free lysates, as well as membrane and cytosolic fractions for more reliable, consistent, and quantifiable immunochemical screening. Eighteen of the ~500 clones screened showed reproducible binding to membrane preparations. Although typical ELISA protocols call for immobilization of antigen by passive adsorbtion of protein antigens to polystyrene plates (which was used in screening most of our clones), we have previously determined that antigens of interest my include glycolipids, which are inefficiently immobilized by standard protocols [4]. Figure 1 compares binding of commercial MAbs against known antigens (Gangl-CK18) as well as selected scFvs (A1-CF10) to fractions of LNCaP cells. These experiments used traditional immobilization (wet) as well as antigen "dry-down" followed by fixation with 1% glutaraldehyde (fixed). As expected, a cocktail of anti-glycolipid antibodies (against G_{D2}, G_{D3} & G_{T1b}: Gangl) bound preferentially to fixed membrane fractions, while an antibody against a cytoskeletal protein (cytokeratin 18; CK18) binds preferentially to wet immobilized cytosolic fraction. Surprisingly, a MAb against a cytoplasmic/nuclear protein (androgen receptor: AR) shows stronger binding to the fixed membrane fraction. All scFvs selected on whole cells preferentially bind to fixed membrane preparations, though several also recognize molecules abundant in cytosolic fractions (A4, G1, TO11C, TO4Q). In almost all cases, fixed preparations yielded higher signal than traditional immobilization. This may be due recognition of relatively rare molecules in the crude preparations, and immobilization of larger amounts of protein when dried and fixed. As expected, an scFv selected against purified ubiquitin binds to both fractions.

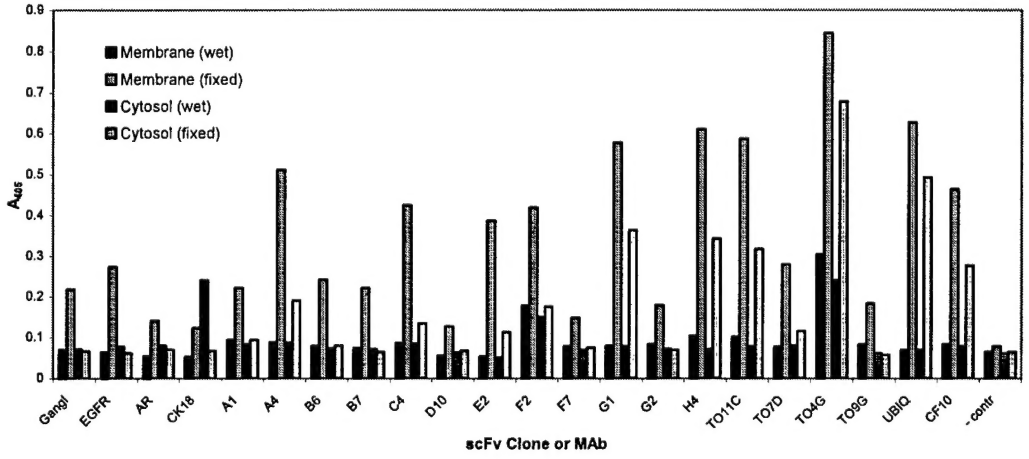


Figure 1: Fixation of crude cellular fractions increases detection of binding

Although several clones show slight differences when membrane preparations from LNCaP and C4-2 cells are compared, none showed the kind of substantial differences that would simplify subsequent analysis (Figure 2).

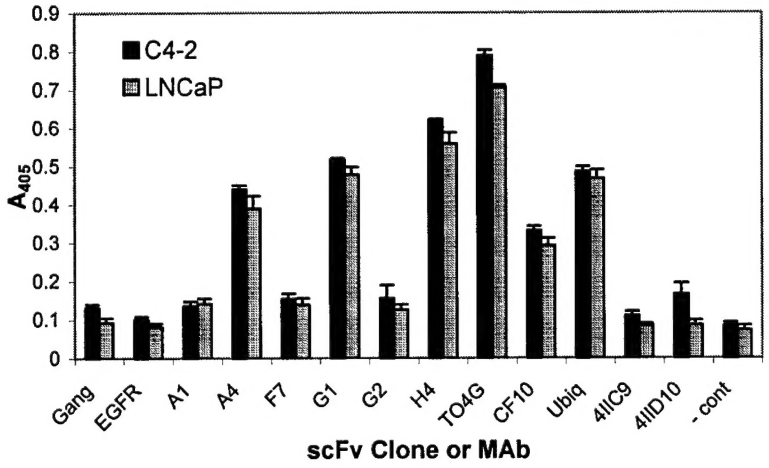


Figure 2. Selected scFv clones recognize antigen present both non-metastatic and metastatic cell membranes.

Work has also continued on cell lines RWPE-1 and RWPE-2. RWPE-1 is HPV-immortalized "normal" prostatic epithelium (non-tumorigenic) and line RWPE-2 is a K-*ras*-transformed (tumorigenic) derivative of RWPE-1 [5]. Collectively, these four lines provide a continuum of properties that reflect progression of prostatic disease from normal androgen-responsive and capable of differentiation into gland-like acini (RWPE-1) [6], through a series of transforming insults to androgen insensitive and highly metastatic (C4-2) [1]. None of the selected scFv clones show significant differences when tested on membrane preparation from the progression series RWPE-1/RWPE-2/LNCaP/C4-2.

Immunoperoxidase staining and cytochemical analysis of a radical prostatectomy specimen using scFv clone CF10 (which was previously selected on RWPE-2 cells) was performed at Emory in conjunction with pathologist Dr. Sharon Lim. This scFv appears to preferentially bind to the basal lamina and blood vessels, and only minimally to luminal epithelial cells (Figure 3). ELISA analysis indicates that it does not bind to collagen I, fibronectin, nor laminin. Since we have not yet isolated phenotype-specific scFvs, no further specimen analysis is anticipate in the coming year.

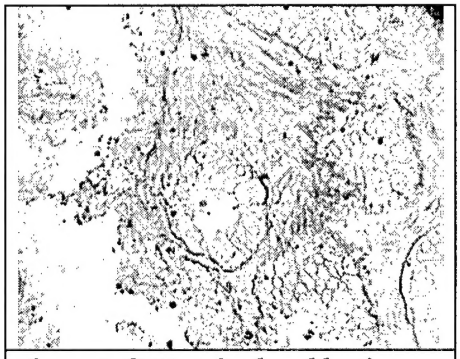
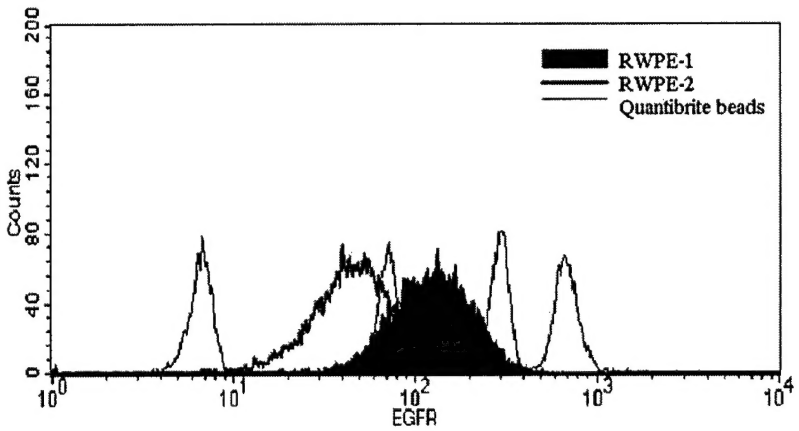


Figure 3: CF10 stains basal lamina. Radical prostectomy specimen stained with crude CF10 scFv, Protein-L-HRP, and DAB.

OTHER APPROACHES

Flow Cytometric Analysis Of Surface Antigens

We have acquired the capability to perform flow cytometry in our lab, and have use this as a tool to quantitatively assess surface antigen expression in PCa cells. We have observed a 2-3 fold decrease in the level of cell surface epidermal growth factor receptor (EGFR) between RWPE-1 (~12,000 molecules/cell) and RWPE-2 (~5000 molecules/cell; figure 4a). This cell surface difference is reflected in the total amount of cellular EGFR, as detected by Western blot (figure 4b), and is not due to differences in subcellular localization of the receptor. Microarray and RT-PCR experiments suggest that this difference is the result of differential protein turnover rather than transcriptional regulation of the EGFR gene. Similar downregulation is seen for the related growth factor receptor Her2/neu, which is frequently expressed in breast and prostate cancers [7].



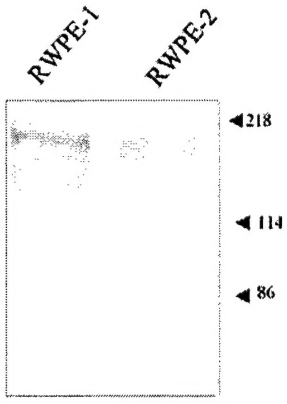


Figure 4. Expression of EGFR is 3-fold repressed in tumorigenic RWPE-2: a) Flow Cytometric analysis. RWPE-1 & RWPE-2 cells were detached, washed, and stained with PE-conjugated anti-EGFR antibody before being run on a BD FACSort flow cytometer. Calibration with Quantibrite beads allowed calculation of the minimum number of EGFR molecules per cell. **b) Immunblot.** Equal amounts of whole cell lysates from RWPE-1 & 2 were probed with anti-EGFR

The level of EGFR expression in both cell lines is decreased at low cell density, implying potential positive autocrine regulation. RT-PCR and microarray analyses suggest that EGFR ligand TGFα is produced in both cells lines. In contrast, only very low levels of expression (~ 500 molecules/cell) are seen in LNCaP and C4-2 cultured under our standard conditions. Others have demonstrated upregulation of EGFR expression in LNCaP cells by androgens, which we do not routinely include in our culture medium [8].

Small GTP-binding proteins like ras require attachment of a isoprenyl group (farnesyl or geranyl geranyl) for activity of the protein; thus, inhibitors of protein prenylation are proposed as anti-cancer therapeutics. Preliminary results suggest that inhibition of protein prenylation with farnesyl transferase inhibitor FTI-276 and geranylgeranyl transferase inhibitor GGTI-298 [9] upregulates EGFR expression in RWPE-2, while having no effect RWPE-1. Thus, activated *ras* appears to increase growth factor receptor turnover. We are investigating both the signal

transduction cascade through which *ras* acts to cause this downregulation, and the consequences of EGFR downregulation for proliferation and tumorigenicity. If this *ras* mediated downregulation of growth factor receptors is a general phenomenon, then EGFR targeted therapies [10] for treatment of cancer with mutant *ras* may be contraindicated.

Scfv Purification And Antigen Characterization

Tomlinson libraries I and J were comprise a single scFv framework in which 18 amino acid residues corresponding to antigen- contact sites were randomized using degenerate oligonucleotides NNS (library I) and DVT (library J), to generate 32 and 8 different codons, respectively. Although equal amounts of each library were mixed for all selections, only 1 of ~ 20 sequenced clones derive from library "J", and all of those from library "I" have at least one amber stop codon. This is permissible since the *E. coli* strain (XL1Blue [11]) used generating phage for selection carries an amber suppressor. Although just more than ½ of clones from library I (18 residues x NNS) theoretically carry at least one amber codon; the apparent enriched frequency among the selected clones suggests that there is a selective advantage for stop codons. A modified protocol for growth of expression libraries including glucose repression, which should limit the induction of the recombinant protein even when scFv-phage are being produced, did not significantly alter this observed bias.

Towards generic biochemical methods characterization of the antigens recognized by recombinant scFvs, we are developing methods for production of scFv in high yield. The purified scFv will be used in several schemes for affinity purification of their cognate antigens from crude lysates of target cells. In addition, the effect on attachment and growth of the target cells will be assessed by addition of pure scFv to culture media. Two clones are being used for development of immunoaffinity antigen characterization methods; Hb5 (which binds to hemoglobin) and one we've selected, designated CF10, that reproducibly binds to whole prostate cell lysates,. A capture ELISA procedure for antigen-independent detection of scFv was developed (plate coated with unlabeled Goat anti-human FAb, detection with Protein L-HRP); thus yields an purification of scFv can be quickly measured. Low level expression of the scFv in the phagmid vector is a bottleneck to the characterization methods. Other investigators have subcloned each scFv gene into alternate expression vectors, but if we ultimately are to examine large numbers of clones, this would be a time-consuming solution to the bottleneck. It was anticipated that *E. coli* strains like BL21 CodonPlus (Stratagene) would produce higher yield than XL1Blue, because it is non-suppressing (eliminating read-through of the amber stop codon between scFv and gene3), absence of the *Lon* protease (decreased protein degradation), and presence of extra copies of rarely expressed tRNA genes. We used site directed mutagenesis to construct a derivative of CF10 where the VH amber codon was replaced with a glutamine codon. Surprisingly, when expressed in XL1B, BL21, and BL21 CodonPlus strains, highest yields of scFv were obtained in XL1B, and no production was detected in the CodonPlus strain. We have purified > 10 μg of CF10 and Hb5 by IMAC, and are preparing to use antigen capture and oriented immobilization [12] on a SELDI chip to characterize antigen by mass spectrometry. Preliminary studies using anti-hemoglobin scFv Hb5 and an Hb5-GFP fusion protein indicate that effective scFv immobilization and affinity purification of antigen can be achieved using this immobilization method (Figure 5).

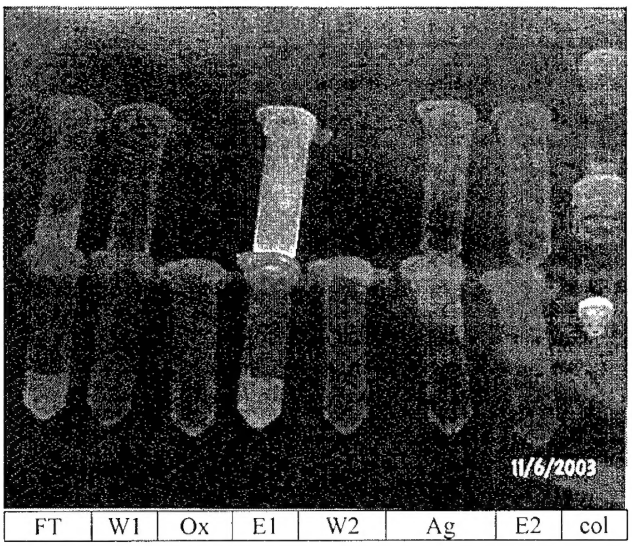


Figure 5: Irreversible immobilization of scFv. Fluorescence image of fractions from Co^{++}-NTA microcolumn purification and immobilization of 6-His tagged HbGFP. Following binding of HbGFP, column was either not treated (top row) or treated (bottom row) with H_2O_2 to oxidize Co^{++} to Co^{+++}. Bound molecules were eluted with 0.25 M imidazole (E1) or 8 M urea (E2). Fractions shown are flow through (FT), Wash 1, 2, (W1, W2), 0.2% H_2O_2(Ox), elutions 1 & 2 (E1, E2), antigen (Ag) or the microcolumn itself (col). When Hb5 was immobilized by a similar procedure, quantitative removal and elution of added hemoglobin antigen from fractions equivalent to Ag & E2 (respectively) was shown by ELISA.

cDNA Microarray Analysis

Examination of differences in gene expression is an alternative approach to discovery of prostate cancer associated antigens. We have probed Affymetrix human U133A microarrays microarrays, carrying 33,000 genes, with cDNA from RWPE-1 and RWPE-2 cells grown under identical conditions. In an initial trial, 730 genes were upregulated and 866 genes were downregulated greater than two-fold. A number of extracellular matrix proteins particularly fibronectin and laminin are among the upregulated genes; this is consistent with observed differences in adhesion and attachment. Several insulin and insulin-like growth factor related genes are also highly regulated. We anticipate repeating these experiments, and comparing the RWPE-1 & RWPE-2 results with LNCaP and C4-2. For further investigation, we will target cell surface antigens, and particularly those related to interactions with the extracellular matrix whose involvement in metastasis and cancer-stroma interactions are probable [13]. This investigation will result in enhanced interaction with the group of Leland Chung. Some of the differentially expressed genes that appear to have transmembrane regions are less well characterized. The sequences of these genes can be analyzed to define probable exposed extracellular domains, and synthetic peptides used to select recombinant antibodies that recognize these epitopes. Collaborative efforts with Emory researchers continue, and have resulted in submission of a manuscript on mutations in mitochondrial DNA.

Although we have discovered differential expression of cell surface associated antigens by a variety of means (membrane extraction & thin layer chromatography of glycolipid gangliosides; flow cytometric staining of EGFR; cDNA microarrray up-regulation of extracellular matrix fibronectin) our recombinant antibody selection methods have not yet revealed even these antigens. Thus, we will optimize the methods for generation of molecular diversity and selection of recombinant proteins for use as affinity reagents, even as we continue the new lines of investigation for discovery and analysis of metastasis associated prostate antigens.

KEY RESEARCH ACCOMPLISHMENTS:

- Selected scFvs that bind to prostate membrane antigens
- Discovered growth factor receptors differentially expressed in prostate cell lines
- Discovered lead genes for tumorigenesis-associated biomarkers; gained expertise in microarray analysis
- Demonstrated feasibility of IMAC-based irreversible immobilization of scFv for purification and characterization of antigens

REPORTABLE OUTCOMES:

Poster Presentations

Williams MNV, Haider J, Jean-Joseph B, Deleon-Mancia M, Flowe C. "K-*ras* Regulation Of Anchorage-Independence And Antigen Expression In Human Prostate Cell Culture." HUPO 2nd Annual & IUBMB XIX Joint World Congress, October 14-18, 2003. Montreal, Canada.

Manuscript Submitted

Stephens KW, Williams MN, Hosseini SH, Kimbro KS, Wallace DC, Wood, WC, Phillips, RF, Lloyd, RR and Flowers LC "Novel mitochondrial mutations in breast cancer" *Submitted to* Mitochondrion

Manuscript In Preparation

Okou DT, Chiang CF, Griffin TB, Verret CR, and Williams MNV. "A Bifunctional Fusion Protein Comprising GFP and a Single Chain Fv" *May 2004 submission, Arch Bioch. Biophys.*

CONCLUSIONS:

Selective procedures that we are using to identify antigens that are expressed on prostate cancer cells have not yet identified metastasis-specific antigens. We will thus extend our selective methods to include other protein scaffold and affinity selection methods Epidermal growth factor receptor appears to be down-regulated in prostate cells transformed with activated K-*ras*; this may have significant therapeutic consequences. We will examine growth factor signal transduction cascades and involvement of extracellular matrix proteins in tumorigenicity and metastasis in the cell lines. Microarray-based cDNA hybridization analysis has yielded candidate biomarkers for prostate tumorigenicity which will be extended to address the metastastic phenotype.

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APPENDICES: None